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(54) Title: PROCESS FOR THE PRODUCTION OF NON-HUMAN EMBRYOS OF HIGH-GENETIC VALUE AND OF PREDETERMINED SEX

(57) Abstract: The process described herein concerns the production of non-human mammalian embryos of predetermined sex, with high-genetic value. The process uses frozen semen, frozen according to EU regulations, obtained from animals selected for their genetic characteristics at Semen Collection Centres, which is thawed and sexed by flow-cytometry. Sexed spermatozoa are used for the *in vitro* fertilisation of oocytes of a compatible animal species for the*in vitro* production of embryos of predetermined sex. The operating conditions of the present process allow the use of frozen semen.

PROCESS FOR THE PRODUCTION OF NON-HUMAN EMBRYOS OF HIGH-GENETIC VALUE AND OF PREDETERMINED SEX

Field of the invention

The technical fields of the invention are the *in vitro* fertilisation techniques for use in the zootechnical industry.

Prior art

The broad diffusion of artificial fertilisation (AF) techniques allowed the zootechnic industry to improve the process for producing breeding animals selected on the basis of useful characteristics. Sex pre-selection techniques represent a further improvement of these procedures favouring either milk or meat production or both, according to the breeder's and to the market's needs. In fact, on the one hand it is possible to improve the selection either on the female line, i.e. for milk production or on the male line, i.e. for meat production, while on the other hand it is possible to control, reduce or even avoid sex-related genetic diseases.

The possibility of semen selection and subdivision on the basis of the presence of different sex chromosomes (a procedure called sexing) is of most interest for the zootechnic world, as it enables the birth planning of the offspring in genetic centres of breeding cows and bulls. However, only with the development of flow-cytometry the process for male gametes pre-selection have been improved and standardised becoming industrially useful.

A flow-cytometer consists of: one or more laser beams that intersect the sample; a hydrodynamic system that conveys the cells sample to be analysed to a flow stream of a liquid, iso-osmotic with the sample; a series of light detectors (photodiodes and/or photomultipliers); a computer for data analysis in real time. The analysis is based on the assessment of the light emitted from the cells under examination once they are intersected by the laser beam. Said cells are previously stained with fluorochromes that, when exposed to coherent laser light, emit a particular fluorescence. Fluorescence is amplified by photomultipliers and computer analyzed, using software suitable for signal optimisation and interpretation. The flow-cytometer, due to its high sensitivity, measures the different amounts of DNA present in spermatozoa X in respect of spermatozoa Y (a difference that varies depending on the species) using dyes, such as

Bisbenzimide Hoechst 33342 which binds to the adenine- and thymidine-rich DNA regions.

The following step, i.e. sorting, allows the particles which are passing into the flow stream and which have been charged positively or negatively depending on their characteristics and to parameters previously set by the operator, to be separated. The sperm cells with a different amount of fluorescence are physically separated by two differently charged plates which attract them on the basis of their charge and which divert them into two different collectors. To check the separation efficiency, the collected and separated material may be re-analysed by letting it flow again in the stream.

By defining the sorting on the basis of the bimodality of the frequency distribution of the spermatozoa DNA content (Johnson L.A. *et al.*, 1989, Biol. Reprod. 41:199-203) it is possible to quantify spermatozoa X and Y and subdivide them into two populations (X and Y). Consequently flow-cytometry proved to be valid not only for spermatozoa analysis but also for their physical separation (Garner D.L. *et al.*, 1983, Biol. Reprod. 28:312-321; Johnson L.A., 1991, Reprod. Dom. Anim. 26:309-314; Johnson L.A. *et al.* 1989, Biol. Reprod. 41:199-203; Morell *et al.*, 1988, Vet. Rec. 122:322-324).

Epi-illuminated (frontally in respect of cells) flow-cytometers are favoured in the DNA study of spermatozoa (Pinkel *et al.*, 1982, Cytometry 1:1-9), whereas those with orthogonal illumination should be adequately modified to make them fit for measuring the light diffracted at a 0 degree angle and not only – as it usually happens - the light refracted at 90 degree angle (Johnson L.A. and Pinkel D., 1986, Cytometry 7:268-273). In fact, in orthogonal illumination flow-cytometers the amount of light refracted at a 90 degree angle, which includes fluorescence, is greater when the laser beam intersects the spermatozoon through its larger surface, and is smaller when the laser beam crosses the spermatozoon through its narrower surface. Since the cells let in a flow stream acquire a rotational motion, which is directly proportional to the flow rate, evidence is provided that the thin portion of the spermatozoa head, when crossed by the laser beam, is predominantly turned towards the detector at a 0 degree angle, with production of a lower amount of light refracted at a 90 degree angle and, consequently, of a

lower amount of fluorescence (Pinkel D. *et al.*, 1982, *ibidem*). Since the flow-cytometric detection is based on the fluorescence levels, the simple intersection of cells in a given position by a laser beam may compensate by defect a different fluorochromatisation level. The problem has been overcome with flow-cytometers which uses epi-illumination (with incandescent-arc light source) because, in this case, spermatozoa, whose conformation is flat, are lit up lengthwise and not crosswise (Pinkel D. *et al.*, 1982, *ibidem*). The French patent FR 2,699,678 discloses a sexing method suitable for the sorting of cells without loading them with an electric charge, i.e. under conditions conceptually different from those described so far, conducted on a proper flow-cytometer (PAS III, Partec). The British patent GB 2,145,112 describes a sexing method based on flow-cytometry which exploits the spermatozoa electric charging, performed in a suitable sheath liquid. The method is set up for fresh semen.

A method of semen sexing by flow-cytometry based on the principle of separation depending on the electric charge, and conducted with a flow cytometer of Epics V type (RTM, Coulter), is described in European patent EP 471,758. The sexing process proposed therein is characterised by a series of modifications to the flow-cytometer (for example to the flow cell needle) which create a flow of oriented cells with the larger surface perpendicular to the laser beam. Furthermore, the orthogonally illuminated instrument is modified so to acquire also the fluorescence from light refracted at a 0 degree angle (normally used for particles dimensional assessment only), by replacing the common photodiode, which cannot detect low fluorescence levels, with a photomultiplier. Said modifications, described in Johnson L.A. e Pinkel D., 1986, Cytometry 7:268-273, refers in particular the modification to the needle point which renders standardisation of the method more problematic. This is the reason why protocols have been drawn up in an attempt to minimise the detections variability. The steps taken concern on one hand the elimination of the spermatozoa rotational motion (achieved through tails elimination) and, to the other hand, the maximisation of the nucleus decompactation, which would allow a more precise measurement of the fluorescence intensity (Metezeau P., 1991, Molecular Reprod. and Develop. 30:250-257).

Therefore the extensive patent and scientific literature published so far evidences that the methods and apparatus used for semen sexing by flow-cytometry are subject to continuous adjustments and improvements for always better standardisation and reproducibility of the results. In many cases their use has been described only for fresh semen.

Summary of the invention

The process of the present patent application describes the separation of frozen semen, according to a different DNA content carried by the X and Y sex chromosomes, by flow-cytometry. The process object of the present application is characterised by specific and critical staining procedures. Sperm separation and collection allow an efficient *in vitro* fertilization (IVF). The process is useful in the zootechnical industry for the production of non-human mammalian embryos of predetermined sex and of high-genetic value. The non-human embryos produced according to the process of the invention are endowed with a high-genetic value, as being obtained from gametes selected on the basis of the genetic characteristics of their donors. This represents a great advancement in the zootechnical industry, especially when focused to the production of progenitor of high-genetic value progeny, for all the breeding and farming animals i.e. the bovine, ovine, equine rodent species, such as cattle, buffaloes, horses, sheep, pigs, mice etc..

According to the process of the present invention, the semen, selected by the genetic characteristics of the animal, is frozen according to standardized procedures laid down by the European Union. It is then carried wherever the production of high-genetic-value embryos is industrially useful and economically profitable. The frozen semen is promptly thawed and stained with a DNA specific fluorochrome such as Hoechst 33342 under conditions allowing the highest spermatozoa vitality. Then, it is separated by flow-cytometry into two spermatozoa subpopulations, one containing chromosome Y and the other containing chromosome X. Depending on the genetic characteristics needed in the breeding centre where the sperm is conveyed, either subpopulation is used for the *in vitro* fertilisation of homospecific oocytes and for the production of high-genetic value non-human embryos of predetermined sex.

Brief description of the figures

Figure 1 shows the cytograms obtained as FL2/FL1 ratio (FL2: 0 degree angle fluorescence; FL1: 90° angle fluorescence), which were obtained to select the region with the highest fluorescence in FL1 and representing the population of spermatozoa which cross the laser beam with their larger surface. The region of interest has been boxed.

Figure 2: Histogram of fluorescence at a 90 degree angle, made on the region previously selected. The gate allowing the assessment only of the spermatozoa crossed by the laser beam in the larger surface and, therefore, with higher FL1, has been marked.

Figure 3: Histogram of fluorescence at a 0 degree angle of spermatozoa with higher FL1 selected by gating. It is shown that the two spermatozoa subpopulations are physically separated. The gate useful for sorting is the one that selects the two bimodal histogram tails of fluorescence in FL2: the tail on the left corresponds to the spermatozoa containing chromosome Y (<DNA content, <fluorescence), and that on the right corresponds to the spermatozoa containing chromosome X (>DNA content, > fluorescence).

Figure 4: Evaluation of the efficiency of the spermatozoa sorting procedure by a genetic-molecular : PCR (polymerase chain reaction). Amplification was performed on sorted samples DNA and with standard samples of Y sorted gametes DNA opportunely contaminated with female DNA, to obtain a standard curve. DNA primers specific for the Y chromosome (BRY4a) were used. After amplification 15 µl of reaction product were electrophoresed for 1 h at 80 Volt on 2% agarose gels and stained with ethidium bromide. Gels were visualized with a UV ligth source, photographed with Polaroid film, and product bands quantified. The band intensities were used to quantify the relative amount of Y chromosomal DNA in each sample compared against the standard curve.

A: DNA extracted from female blood; B: DNA extracted from female blood + DNA extracted from a pool of semen (corresponding to an enrichment of gametes carrying the Y chromosome of 10%); C: DNA extracted from female blood + DNA extracted from a pool of semen (enrichment of 20%); D: DNA extracted from female blood + DNA extracted from a pool of semen (enrichment of 30%); E: DNA

extracted from female blood + DNA extracted from a pool of semen (enrichment of 40%); F: DNA extracted from a pool of semen (50% of Y); G: DNA extracted from sorted semen (X spermatozoa); H: DNA extracted from sorted semen (Y spermatozoa); I: negative control (water).

Detailed description of the invention

Object of the present patent application is a process for sexing frozen semen by flow-cytometry. The new process is characterised by specific and critical staining, separation and collection conditions, which will be described hereinafter in detail. The semen sexed under the conditions described in the present patent application is endowed with a fertilising capacity useful for an effective *in vitro* fertilization (IVF) for the production of non-human mammalian embryos of predetermined sex (e.g. of cattle, buffaloes, horses, sheep, pigs, rodents).

According to a first embodiment, the invention describes a process for the production of non-human high-genetic value embryos of predetermined sex derived from gametes of breeding mammalian species, which comprises the following steps:

a) thawing out a frozen solution of genetically selected semen and diluting it;

b) staining the spermatozoa with a DNA specific fluorescent dye;

c) diluting out the sample;

d) sorting the stained spermatozoa population by flow cytometry into a spermatozoa subpopulation containing chromosome X and into a spermatozoa subpopulation containing chromosome Y;

e) *in vitro* homologous fertilisation of oocytes with one or the other spermatozoa subpopulation;

f) incubation of fertilised oocytes into a mammalian culture medium.

According to a particular embodiment of the present invention step a) of the process may be optionally preceded by a freezing step usually performed according to process well known in the art such as those officially acknowledged by the European Union. The semen is derived from animals which have been selected on the basis of their genetic characteristics and/or on the genetic characteristics of their progeny, as for example semen derived from bulls which are genetic fathers of cows producing very high amounts of milk and/or which

were found, when tested, to be free from genetic diseases like the biochemical defect BLAD (Bovine Leukocyte Adhesion Deficiency) and/or DUMPS (Deficiency of Uridine Monophosphate Synthase) in Halstain breed. It may be derived from Semen Production Centres, were it is usually aliquoted, stored and frozen in 0.25-0.5 ml *paillettes* (also known as "French" straws). The frozen semen is conveyed, at a constant and controlled temperature, wherever the production of high-genetic-value embryos is industrially useful and economically profitable, i.e. to various breeding centres, potentially without any limit of time and place.

According to the process of the invention, in step a) of the process the frozen semen is promptly thawed, preferably in a thermostated water bath at 37°C for at least 30", then it is preferably diluted with a buffer solution at a preferred spermatozoa concentration comprised between 15 and $25x10^6$/ml, more preferably $18\text{-}22x10^6$ spermatozoa/ml. The dilution buffer is preferably chosen according to the extender used for semen freezing. In fact a citrate buffer is preferred when a solution such as the Laiciphos buffer containing milk-derived proteins is used as extender in the preparation of the frozen sperm, while a TALP (Tyrode's Albumin Lactate Pyruvate) Ca^{++} and BSA free solution (whose composition will be specified ahead) is preferred when buffer solutions containing soya- (such as Biociphos) or egg yolk-derived proteins are used for the same purpose. A commonly used citrate buffer has the following composition: 109 mM tribasic bihydrated sodium citrate and 2.4 mM citric acid, in water.

The process of the present invention utilizes thawed semen with a progressive motility (PM) not lower than 40% (per cent amount of spermatozoa with VAP (Velocity Average Path) > 25 μm/sec and STR (Straightness) > 0.8, according to WHO's parameters (World Health Organization, 1992, Laboratory manual for the examination of human semen and sperm-cervical mucus interaction).

According to the process of the invention, spermatozoa are stained in step b) of the process by incubation in a DNA specific fluorochrome dye solution. The dye used is Bisbenzimide Hoechst 33342, used at conditions able to maintain the spermatozoa vitality at an optimal level. This condition is achieved by the use of Bisbenzimide Hoechst 33342 in concentration of at least 50 μg/ml, preferably comprised between 70 and 180 μg/ml, even more preferably comprised between

80-120 μg/ml and for a time not longer than 30 minutes, preferably comprised between 10 and 20 minutes, more preferably 15 minutes, at a temperature ranging from 30°C to 38°C, more preferably 34°C-36°C, most preferred 35°C.

According to a preferred embodiment the staining is performed by adding the fluorochrome at a final concentration of 100 μg/ml at the solution containing the spermatozoa, for a time of about 15 minutes, at a temperature of 35°C. After labelling, sperm cells may be optionally washed with the same buffer used for the previous dilution. The sample is then further diluted with a protein-free buffer or with isotonic solutions. According to a preferred embodiment of the invention, in step c) is used the same buffer as in step a), that are preferably a citrate buffer and/or an "enriched buffer solution", such as the TALP Ca^{++} and BSA free solution. By the use of the preferred solutions the total dissolution of the freezing extender is observed and a better definition of the spermatozoa populations to be read and sorted is obtained during flow-cytometry.

The composition of the TALP solution for in vitro fertilisation (IVF) is known in the art (see Ball GB et al., Biol Reprod, 1983, 28:717-725). In the process of the invention, this solution is called TALP IVF and is the buffer of choice for *in vitro* fertilisation. The solution, as used in the method of the invention also for purposes different from IVF techniques, is modified and Calcium and/or BSA are not added. Other slight variations are introduced in the composition to maintain the optional osmolarity (comprised between 270-300 mOsm). When both BSA and calcium are lacking the solution is therefore called TALP Ca^{++} and BSA free and has essentially the same composition as the TALP IVF, and is perfectly compatible in case of mixing of the two.

When used as diluting or sheath fluid for spermatozoa separation by flow-cytometry TALP lacks BSA and calcium. The composition of the preferred TALP Ca^{++} and BSA free version is here reported: NaCl 6000 mg, KCl 230 g, Na_2HPO_4 40 mg, $MgCl_2 \cdot 6H_2O$ 310 mg, hepes 4760 mg, sodium lactate 60% 3700 μl, kanamycin 75 mg, sodium piruvate 110 mg, PVA (polyvinyl alcohol) 1000 mg, $NaHCO_3$ 420 mg, NaOH 420 mg, sterile H_2O 1000 ml; pH 7.4, with an osmolarity comprised between 270-300 mOsm, preferably comprised between 280-290 mOsm. It is known that other slight variations of this solution may be introduced.

The sample is diluted at a preferred final ratio of 3:1 (sample:buffer). It is known in the art, however that other buffer solutions may be used to dilute the sample, as i.e. PBS or Tyrode's, according to the preferences related to the extender used for initial semen freezing, with the provision that no proteic component is added. As in the previous dilution, preferred solutions are citrate buffer or TALP Ca^{++} and BSA free buffer, by the use of which the total dissolution of the freezing extender is observed. By the use of the preferred diluting solutions a better definition of the spermatozoa populations to be read and sorted is obtained.

Sperm separation (sexing) is carried out by flow-cytometry with orthogonal illumination on apparatuses modified according to Johnson L.A., Pinkel D. (1986) Cytometry 7:268-273, e.g. the Epics V (Coulter) instrument. The method of the invention preferably employs the Becton-Dickinson FACS Vantage SE equipped with: i) a laser for the emission of UV light; ii) a MacroSORT™ head supporting a beveled needle as described in Johnson L.A., Pinkel D. (1986) Cytometry 7:268-273; iii) two photomultipliers for fluorescence detection at 0 (an additional standard kit) and 90 degree angles, and suitable filters; iv) preferably the Turbo Sort Plus Option, consisting of a special fluid and electronic system that allows to achieve sorting pressure of up to 60 p.s.i. and sample flow rate of up to 40.000 particles/sec.

The modifications to the flow-cytometer (for example the one to the flow cell needle) create a flow of oriented cells with the larger surface perpendicular to the laser beam. The instrument with orthogonal illumination is modified to acquire also the fluorescence from light refracted at a 0 degree angle (normally used for particles dimensional assessment only), by replacing the common photodiode, which cannot detect low fluorescence levels, with a photomultiplier.

Said modifications allow to increase the apparatus sensitivity: in particular the beveled needle allows to reduce or to eliminate the spermatozoa rotational motion, while the addition of a photomultiplier at 0 degree angle allows a more precise measurement of the fluorescence intensity. The addition of the Turbo Sort Plus Option is however optional, although preferred, allowing only to fasten the sorting procedure and to obtain a high number of sorted sperm cells/second.

The efficiency of the instrument is further optimized by treatment of the sample

according to the process.

After dilution the sample is made to flow in the cytometer (step (d) of the process), where evaluation of the DNA content of the cells and sorting is performed. The solution used as entraining or "sheath" fluid is a protein-free buffer, preferably the same buffer used for the next step of *in vitro* fertilization (or a buffer fully compatible with it). An "enriched buffer solution", such as the TALP Ca^{++} and BSA free solution is preferably used. The composition of the TALP Ca^{++} and BSA free solution has been reported above. This solution allows optimal results both in terms of flow stability and spermatozoa vitality and is usually the solution of choice (with the above mentioned modification on calcium and BSA concentrations) for the fertilisation step. However, other buffer solutions may be used as sheath fluid, i.e. PBS, Tris etc., provided that they do not contain any proteic component.

As said above, sorting is performed by the flow-cytometer on the basis of the different amount of DNA present in spermatozoa carrying chromosome X with respect to spermatozoa carrying chromosome Y. This difference varies depending upon the mammalian species and is anyhow easily detectable by the instrument modified as said above.

According to a preferred embodiment, the process of the invention is performed on bovine gametic cells.

Physical separation of the two subpopulations of gametic cells (spermatozoa), carrying one or the other sex-chromosome, also known as "sorting", allows the cells which are charged positively or negatively depending on their characteristics and according to parameters previously set by the operator, to be collected separately. The separation is carried out through the "gating" procedure, according to defined parameters and separates cells with a different amount of fluorescence: two differently charged plates attract the particles on the basis of their charge and divert them to two collectors. The definition of the sorting parameters on the basis of the bimodality of the frequency distribution of the spermatozoa DNA content (Johnson L.A. *et al.*, 1989, Biol. Reprod. 41:199-203) for spermatozoa carrying the chromosome X and for those carrying chromosome Y allows their subdivision into two populations. According to the methods of the invention the sorting process is carried out preferably using a flow of approx. 25,000/30,000 cells/sec, when the

Turbo Sort is used . Alternatively, when the Turbo Sort device is not used, the flux is approx. 1,500 cells/sec.

The fluorescence of each gametic cell is detected by causing each single spermatozoon to pass through a channel illuminated by UV light, preferably set at a wavelength comprised between 333 to 364 nm; the signal obtained is diverted to two different photomultipliers (FL1 and FL2).

The fluorescence signal from the samples is preferably acquired and displayed by the software CellQuest run on MacOS 7.5.3. operating system. The sperm, separated into the two populations X and Y in TALP Ca^{++} and BSA free buffer, is preferably collected in protein-coated test tubes, such as those obtained by incubating them in a TALP Ca^{++} free (standard) solution containing 5% BSA, for at least 8 hrs.

Spermatozoa are collected, optionally concentrated, e.g. by centrifugation at 400xg, and used for the *in vitro* fertilisation (step (e) of the process of the invention) on primary non-mature (non-ovulated) oocytes. The oocytes are obtained, e.g. by *in vivo* ovum pick-up or from freshly slaughtered animals and are matured *in vitro* using suitable media, such as the commercially available M199, added with hormones and foetal calf serum in controlled temperature and atmosphere conditions. *In vitro* "matured" oocytes may be optionally treated with hyaluronidase to remove contaminating or "coating cells".

According to a preferred embodiment, the *in vitro* fertilisation (step (e) of the process) is carried out according to procedures known in the art: a particularly preferred method is the one described by Galli C. *et al.* in Animal production and Biotechnology, Proceedings of the 19th International Symposium on Zootechnics, 1994, 185-189. According to said method, oocytes are incubated in a medium suitable for spermatozoa capacitation and for fertilisation, such as TALP IVF, whose composition is known in the art and is here reported for completeness: TALP IVF (In Vitro Fertilsation): NaCl 620 mg, KCl 23 mg, Na_2HPO_4 4.7 mg, $MgCl_2 \cdot 6H_2O$ 10 mg, $NaHCO_3$ 220 mg, $CaCl_2 \cdot 2H_2O$ 39 mg, kanamycin 7.5 mg, 60% Na lactate 186 µl, sodium piruvate 11 mg, BSA fraction V (fatty acid free) 600 mg, heparin 0.12 mg, sterile H_2O 100 ml; pH 7.3-7.4, mOsm=270-300.

The preferred ratio between sexed spermatozoa and oocytes is 5000:1

(sperm/oocytes), in a final volume of 50 µl. Incubation is performed for 18 hrs in an incubator at 5% CO_2/air, according to methods well known in the art.

After this incubation time the fertilized eggs are transferred into a cell culture medium such as SOF or M199, until the blastocyst stage is reached. However other commercially available media suitable for embryo development may be used for this incubation. The non-human embryos may be used at the blastocyst stage for the implatantion into surrogated mothers or may be frozen and used in any other moment and in any other place of interest.

A further object of the present invention is represented by high-genetic value embryos, of predetermined sex, as produced in step f) of the process, obtainable by the process according to the invention. They may be used as such or after freezing. It should be noted that the values of these embryos are not limited to the single individual generated by the IVF but also, and more importantly, to the progeny that this single individual will generate and which will carry the genetic characteristics which have been selected for.

As said previously, according to a preferred embodiment of the invention, step f) is followed by an optional stage of freezing of the embryo, performed according to known protocols.

The convenience of the process of the invention, according to this preferred embodiment, is that all the sexing procedure and the in vitro fertilisation operations are performed into a laboratory fully equipped for both of them and they don't need to be performed on site in the breeding center usually equipped only for implantation of the embryo.

Alternatively to the growth in cell culture medium, the fertilised bovine oocyte is co-cultured on a cell monolayer, in the particular and preferred case of bovine oocytes, derived from bovine tubal epithelium.

The embryo obtained may be frozen or directly re-implanted in substitute mothers for the later development growth or it may be analysed to confirm the efficiency of sperm sexing.

The method, object of the present invention, is preferably applied to the bovine species, but can be advantageously applied also to any other breeding mammalian species and in particular to ovine, equine and rodent species, such as:

sheeps, buffalos, horses, goats, pigs, rodents, mice.

The method of the present invention is effective for the production of embryos of predetermined sex of non-human mammalian species from frozen semen. The semen is selected on the basis of useful genetic characteristic of the individual or of the progeny derived and has therefore a high-genetic value. The process of the invention allows the choice of the individual carrying the useful genetic characteristics without time and geographical limitations, because of the use of frozen semen. The possibility to sex frozen semen allows the more efficient production of progeny aimed at particular purposes in the zootechnical industry or addressed to particular production lineage (i.e. female for milk production or male for meat production).

Therefore the method described herein, allowing the use of frozen semen, represents an important technical achievement over the prior art methods where sexing is performed on fresh semen. In fact, in the method of the invention, potentially any semen with the desired characteristics can be used in any country and at any time, i.e. potentially without any time and place limit imposed by the use of fresh semen.

The method of the invention allows a fertilisation efficiency still suitable for industrial purposes. The embryos obtainable by the process of the invention, have a high-genetic value, as they are derived from genetically selected individuals according to characteristics useful in the zootechnical industry and more importantly, they can be produced entirely in the laboratory where they can be frozen and used in the breeding centre.

EXPERIMENTAL PART

The following examples show some of the practical applications of the process of the invention without representing any limitation.

Instrumentation

The FACS Vantage *SE* (Becton-Dickinson) is equipped for the sexing procedure with the following accessories: 1) Water-cooled argon laser, Coherent Innova 300C, with 333.6-363.8 nm bandpass filters; 2) Detectors: the photodiode is replaced by a photomultiplier (PMT) for the detection of fluorescence at a 0 degree angle (FL2); No. 2 Hamamatsu photomultipliers for the simultaneous detection of

at a 90 degree angle scatter light and fluorescence emission (FL1); 3) Filters: 8/90 scatter dichroic filter, 488/10 scatter bandpass filter, 560 shortpass dichroic filter, 424/44 bandpass filter for FL1 (UV), 424/44 bandpass filter for FL2 (UV); 4) Turbo Sort Option, consisting of a special fluid and electronic system that allows to achieve sorting pressure of up to 60 p.s.i. and sample flow rate of up to 40.000 particles/sec; 5) Software: CellQUEST for data acquisition and analysis, on MacOS 7.5.3. operating system.

The main modifications to the Instrument with regard to the protocol of semen sexing were:

1. an additional standard kit, Forward Scatter Photomultiplier, whereby the photodiode collecting the light signal at a 0 degree angle (forward scatter) is replaced by a *photomultiplier* collecting and completing all fluorescence data emitted from the spermatozoa intersected by laser beam;

2. the installation of a modified needle, whereby a greater quantity of spermatozoa can be oriented in the same direction. The needle is inserted in a new MacroSORT™ head.

The aforesaid modifications to the FACS Vantage are described in: Johnson L.A. and Pinkel D. in *Cytometry* 7:268-273, 1986; the modified items are available on the market as FACSVantage (Becton-Dickinson) additional kits.

The modified instrument provides a cytogram which visualizes the different spermatozoa sub-populations obtained when the gametes, which have acquired a different orientation, flow through a laser beam. We were interested in the subpopulation that allows a more accurate analysis of the bimodality due to the different DNA content, since the spermatozoa are intersected by the laser beam in a perpendicular direction.

Solutions used

Bisbenzimide Hoechst 33342 stock solution (5 mg/ml): H_2O or Tyrode's solution 400 µl, Hoechst 33342 (Sigma) 2 mg. The solution was prepared and divided into aliquots which were stored at -20°C.

TALP Ca^{++} and BSA free: NaCl 6000 mg, KCl 230 mg, Na_2HPO_4 40 mg, $MgCl_2 \cdot 6H_2O$ 310 mg, hepes 4760 mg, 60% sodium lactate 3700 µl, kanamycin 75 mg, sodium piruvate 110 mg, PVA 1000 mg, $NaHCO_3$ 420 mg, NaOH 420 mg,

sterile H_2O 1000 ml; pH 7.4 ; mOsm = 284. The solution was prepared and aliquoted in 25 ml stock and stored at +4°C.

TALP IVF (In Vitro Fertilsation): NaCl 620 mg, KCl 23 mg, Na_2HPO_4 4.7 mg, $MgCl_2 \cdot 6H_2O$ 10 mg, $NaHCO_3$ 220 mg, $CaCl_2$ $2H_2O$ 39 mg, kanamycin 7.5 mg, 60% Na lactate 186 µl, sodium piruvate 11 mg, BSA fraction V (fatty acid free) 600 mg, heparin 0.12 mg, sterile H_2O 100 ml; pH 7.3-7.4, mOsm=275. The solution was sterilised with 0.22 µm filter plus prefilter and was aliquoted in 1 ml stock and stored in liquid nitrogen.

TALP wash: NaCl 7300 mg, KCl 235 mg, Na_2HPO_4 47 mg, $MgCl_2 \cdot 6H_2O$ 100 mg, $NaHCO_3$ 168 mg, hepes 2380 mg, 60% sodium lactate 1900 µl, kanamycin 75 mg, sodium piruvate 110 mg, $CaCl_2 \cdot 2H_2O$ 390 mg, BSA fraction V 6000 mg, sterile H_2O 1000 ml; pH 7.3-7.4, mOsm=280. Aliquoted in 25 ml stock and stored at +4°C.

SOF: $CaCl_2$ $2H_2O$ 25.1 mg, $MgCl_2 \cdot 6H_2O$ 10 mg, NaCl 580 mg, KCl 53.84 mg, KH_2PO_4 16.2 mg, $NaHCO_3$ 220 mg, sodium piruvate 3.6 mg, hepes 476 mg, penicillin 6.3 mg, streptomycin 5 mg, 60% sodium lactate 47 µl, D-glucose 27 mg, BSA fraction V (fatty acid free) 1600 mg, glutamine 14.6 mg, non-essential amino acids 1 ml, essential amino acids 2 ml, sterile H_2O 97 ml; pH 7.3-7.4, mOsm=280. Sterilised with 0.22 µm filter plus pre-filter. Aliquoted in 0.5 ml stock and stored in liquid nitrogen.

Lacmoid 1%: 100 mg Lacmoid, sterile H_2O 5.5 ml, glacial acetic acid 4.5 ml. Filtered with 0.8 µm filter.

Example 1: Vitality and dilution tests on Hoechst 33342-labelled samples

Tests were conducted with various concentrations of Bisbenzimide Hoechst 33342: final 5 µg/ml, final 10 µg/ml and 100 µg/ml, with incubation times of 15, 60, 120, 240 e 300 min. The vitality of spermatozoa thawed as described in Example 2 was assessed after different incubation times, at different final dye concentrations: vitality was the highest after a 15-min staining with 100 µg/ml Bisbenzimide Hoechst 33342.

The sample of stained spermatozoa was diluted with various solutions, such as PBS, Tyrode's solution, citrate buffer (109 mM tribasic bihydrated sodium citrate and 2.4 mM citric acid) and TALP Ca^{++} and BSA free.Only these last two proved

to be capable of completely dissolving the freezing extender, such to obtain an optical clearance allowing a better definition of the signal from the populations to be sorted by flow-cytometry.

Various solutions were tested as sheath fluid: i.e. PBS, Tris pH 7, and TALP Ca^{++} and BSA free, pH 7.4. The last solution gave good results in terms of flow stability and spermatozoa vitality and because it did not interfere in the *in vitro* fertilisation (IVF) step in which TALP was always used.

The TALP Ca^{++} and BSA free, pH 7.4 solution and the citrate buffer were not added with BSA; this allowed a better flow stability with consequent improvement of the sorting condition and results. In fact, spermatozoa with sticking heads (doublets), which indicate alterations of the cell membrane, were detected only in the presence - and not in the absence - of BSA.

The sorted sperm was collected according to different methods which were compared for the recovery efficiency: a) in test-tubes with extender based on Tris and egg yolk with or without BSA or in BSA-coated (saturated) test-tubes incubated overnight with TALP Ca^{++} and BSA free pH 7.4, added with 5% BSA. The best results were obtained by collecting the spermatozoa in TALP Ca^{++} and BSA free, pH 7.4, in test-tubes saturated with BSA, which maintains the spermatozoa vitality at an optimal level and does not interfere with the successive *In Vitro* Fertilisation steps. Conversely, the methods known in the art (e.g. with extender based on Tris and egg yolk with or without BSA) maintain a good vitality of spermatozoa, but do not allow an easy recovery of same in the first IVF steps.

Example 2: Sperm sexing and separation of two subpopulations carrying chromosomes X or Y

Preparation of thawed samples

The frozen bovine semen was thawed in a water bath thermostated at 37°C for 2 min. The kinetic parameters of an aliquot of at least 200 spermatozoa was assessed by the system CASA Hamilton Thorne mod. IVOS set as follows: frame acquired = 30, minimum contrast = 15, minimum cell size = 25, threshold straightness = 80, medium VAP cut-off = 25.0, low VAP cut-off = 24.9, low VSL cut-off = 20.0, non-motile head size = 60, non-motile head intensity = 50, 0.19 < static size limits < 5.55, 0.52 < static intensity limits < 10.00, 0 < static elongation

limits < 60.

The sexed semen was used for the following step on flow-cytometry only if its progressive motility was 40 % at minimum (measured as per cent amount of spermatozoa with VAP (Velocity Average Path) > 25 μm/sec and STR (straightness) > 0.8),

The semen was diluted in a citrate buffer (109 mM tribasic bihydrated sodium citrate and 2.4 mM citric acid) or in TALP Ca^{++} and BSA free to a spermatozoa concentration of $20x10^6$/ml. Then an aliquot of Bisbenzimide Hoechst 33342 solution (5 mg/ml in Tyrode's buffer/H_2O) was added to obtain a final concentration of 100 μg/ml. The sample was then incubated at 35°C for 15 min. For fluorescence assessment and for the successive spermatozoa separation, the sample was caused to flow in the instrument using TALP Ca^{++} and BSA free, pH 7.4, as sheath liquid.

Fluorescence analysis

By means of an argon laser, Innova 300C, rated at 150 mW, fluorochromatised spermatozoa were excited by 333.6-363.8 nm UV light.

From the cells caused to flow at 25.000-30.000 cells/sec, the fluorescence data, at 0 and 90 degrees, were acquired in 1024-channel histograms and cytograms. In particular the following was assessed:

a) cytogram FL2 (fluorescence at 0°)/FL1 (fluorescence at 90°) for the selection of the higher-fluorescence region in FL1 (spermatozoa passing through the laser beam with their larger surface) (figure 1);

b) histogram of fluorescence in FL1, at a 90 degree angle, made on the region previously selected. The gate allowing the assessment only of the spermatozoa crossed by the laser beam in the larger surface and, therefore, with higher FL1, has been marked in figure 2.

c) histogram of fluorescence in FL2 of the subpopulation defined above for bimodality control (figure 3)

It is thus possible to collect and integrate all data concerning the fluorescence emitted in the various direction from the spermatozoa crossed by the laser beam in their larger surface (length-wise).

The definition of the subpopulations to be sorted was carried out through gates

which select the two tails of the bimodal histogram of fluorescence in FL2: the tail on the left corresponds to the spermatozoa containing chromosome Y (lower DNA content and, therefore, lower fluorescence); the tail on the right corresponds to the spermatozoa containing chromosome X (higher DNA content and, therefore, higher fluorescence).

Collection of spermatozoa subpopulations X and Y

The spermatozoa sorted according to the procedure described above and collected in BSA-lined test-tubes, were concentrated by centrifuging at 400 g for 10 min. Once the surnatant was removed, the pellet volume was measured.

Example 3: Control of the efficiency of Y and X sorting

The semen sexing was checked on the sorted material by cytometric re-analysis or by genetic-molecular methods.

In the first case, the sexed material collected was re-analysed by flow cytometry. By particular computer software, it is possible to overlap the histograms of fluorescence in FL2, obtained from spermatozoa X and Y. The results obtained by this method indicate that in the sorted subpopulations the enrichment of spermatozoa containing the X or Y chromosome is always higher than 75%.

A semi-quantitative approach based on PCR (polymerase chain reaction) was also used to confirm the efficiency of sorting using DNA primers specific for the Y chromosome (BRY4a, see Peura et al., Theriogenology, 1999, 35:547 -555).

The Y chromosome-specific primers corresponded to nucleotides 1159-1178 (5' primers) 5'-CTCAGCAAAGCACACCAGAC-3' and 1439-1459 (3' primers) 5-'GAACTTTCAAGCAGCTGAGGC-3'. Image analysis of the fluorescent intensity of PCR amplified Y chromosome-specific DNA was used to quantify the relative amount of y chromosomal DNA in each sample. Fluorescent intensity of each sample was compared with that of a reference sample prepared from pooled unsexed ejaculate (pooled sperm). The standard curve consisting of 30 ng/µl of female DNA (only XX) diluted with 30 ng/µl pooled sperm DNA in different ratio (see Table 1).

Table 1. Preparation of the standard for PCR amplification.

% Y	DNA from unsexed sperm (30 ng/µl) + DNA from female blood (30 ng/µl)	corresponding lane in Figure 4
0	0 + 5	A
10	1 + 4	B
20	2 + 3	C
30	3 + 2	D
40	4 + 1	E
50	5 + 0	F

The 50 µl pcr reaction contained 30 ng of DNA template, 1x PCR buffer (Perkin Elmer), 200 µM each dNTP, 1,5mM $MgCl_2$, 250 mM of each primer and 1.25 U of Taq Gold polymerase (Perkin Elmer).

The amplifications were carried out in a GeneAmp pcr System 9700 (PE Applied Biosystem). The samples were denaturated at 95 °C for 10 minutes followed by 20 cycles consisting of denaturation for 15 seconds at 95 °C, annealing for 15 seconds at 65 °C and primer extension at for 72 °C for 1 minute. After the last cycle the samples were incubated for a further 5 minutes at 72°C. After amplification 15 µl of reaction product were electrophoresed for 1 h at 80 Volt on 2% agarose gels, stained with ethidium bromide.

Gels were visualized with a UV light source, photographed with Polaroid film, and product bands quantified. The band intensities were used to quantify the relative amount of Y chromosomal DNA in each sample compared against the standard curve. The data are shown in figure 4. As an indicative example see sample G were it is shown that in the subpopulation sorted for the X chromosome the enrichment of spermatozoa is higher than 90% as compared with the standard B where a 10% of Y DNA "contamination" is present.

The results obtained using both methods show that in the sorted subpopulations the enrichment of spermatozoa containing the X or Y chromosome is always

higher than 75% confirming the efficiency of the method.

Example 4: *In vitro* fertilisation and pre-implant growth of fertilised oocytes

In vitro fertilisation of oocytes matured *in vitro* was substantially performed according to Galli C., Aleandri R., Lazzari G., Animal production and Biotechnology, Proceedings of the 19th International Symposium of Zootechnics, 1994, 185-189. From a 500 µl TALP IVF drop, allowed to equilibrate in 5% CO_2 incubator at 38°C for at least 30 min, an aliquot was taken equal to the sexed spermatozoa pellet volume (measured as described in Example 2) and replaced by it. Said solution was used to prepare 50 µl drops, which were covered with hot mineral oil, previously equilibrated in 5% CO_2 incubator at 38°C overnight. Five mature oocytes, denuded of the *cumulus oophorus* by hyaluronidase, were added to each drop.

After incubation in 5% CO_2 incubator at 38°C for 30 min., the oocytes were added to TALP wash and freed from the spermatozoa and cells of the "corona radiata" by mechanical vortex motion.

A few of said fertilised oocytes were put on an ethanol-washed slide, closed with a cover glass to check whether fertilisation had taken place. The slides were allowed to stand overnight in a fixative (ethanol/acetic acid 3:1). Then they were dyed by capillarity with 1% lacmoid and promptly rinsed with 45% acetic acid. If fertilisation took place, the two male and female pronuclei and the penetrated spermatozoon tail can be viewed through a microscope. Otherwise, i.e. in the case of non-fertilised oocyte, the maturation of same is proved by the presence of a metaphase with a polar globule.

Once oocytes were fertilised and denuded by vortex motion, they were cultured in SOF. To this end, two 20µl microdrops of said medium covered with mineral oil had to be prepared one day before in suitable containers (four-wells). Microdrops had to be equilibrated for one day in a 5% CO_2 and 5% O_2 incubator at 38°C. Denuded oocytes were added to the first wash drop; the still uncleaved oocytes (those being cleaved after 18 hrs indicate a parthenogenic activation) were soon transferred into the second drop (approx. 20 oocytes/drop) and fed to an O_2 incubator. On the following day, the first cleavage of fertilised oocytes could already be observed; the uncleaved ones were transferred to the lateral wash

drop. Two days later, the medium was replaced by adding the culture drop with 20µl SOF, equilibrated in an incubator, and by sucking an equal amount of it. After two further days of culturing in SOF, this medium was replaced, according to the same procedure, by equilibrated medium M199. This medium will support the embryos development to the blastocytes stage, which will be reached on the two following days.

The results obtained from the in vitro fertilisation IVFs are shown in table 2.

Table 2

N°.spermatozoa/oocytes	N°. oocytes	No. fertilised oocytes	% fertilisations
Sperms X	194	70	36.1
Sperms Y	207	85	41.1

CLAIMS

1. Process for the production of high-genetic value embryos of predetermined sex derived from gametes of non-human mammalian species, comprising the following steps:

a) thawing a frozen solution of genetically selected spermatozoa and diluting it;

b) staining the spermatozoa with a DNA specific fluorescent dye;

c) diluting out the stained sample;

d) sorting the stained spermatozoa population by flow-cytometry into a spermatozoa subpopulation containing chromosome X and into a spermatozoa subpopulation containing chromosome Y, using as a sheath fluid a protein free solution;

e) *in vitro* homologous fertilisation of oocytes with one or the other spermatozoa subpopulation;

f) incubation of fertilised oocytes into a mammalian cell culture medium.

2. The process according to claim 1, wherein dilutions in step a) and in step c) are performed using protein free solutions

3. The process according to claim 2, wherein said protein free solution is a citrate buffer or TALP Ca++ and BSA free

4. The process according to claim 1, wherein in step d) said protein-free sheath fluid is essentially the same buffer used for *in vitro* fertilisation.

5. The process according to claims 1-4, wherein spermatozoa DNA staining in step b) is performed with the fluorochrome bisbenzimide Hoechst 33342.

6. The process according to claim 5 wherein said staining is obtained after incubation in a solution comprising bisbenzimide Hoechst 33342 at a concentration higher than 50 μg/ml, for a time shorter or equal to 30', at a temperature comprised between 30°C and 38°C.

7. The process according to claim 6, wherein said concentration is comprised between 70 and 180 μg/ml.

8. The process according to claim 6-7, wherein the bisbenzimide Hoechst 33342 concentration is comprised between 80 and 120 μg/ml, said temperature is comprised between 34°C and 36 °C and said time is comprised between 13 and 17 minutes.

9. The process according to claim 8, wherein the thawing in step a) is performed by incubation of said frozen solution at a temperature of 37°C for a time of at least 30" (minute second).

10. The process according to claim 1, wherein the dilution in step a) is such as to obtain a spermatozoa concentration comprised between 15 and $25x10^6$ spermatozoa/ml.

11. The process according to claim 1, wherein staining in step c) of the process is performed on spermatozoa having a progressive motility not lower than 40%.

12. The process according to claims 1-11, wherein said sorting performed by flow cytometry in step d) of the process is carried out on the Becton-Dickinson FACS Vantage SE.

13. The process according to claim 1, wherein incubation of the fertilized oocyte in step f) of the process is performed until the oocyte has reached the blastocyst stage.

14. The process according to claims 1-13 wherein step f) of the process is followed by freezing of the fertilized oocyte or blastocyst.

15. The process according to claim 1, wherein the non-human mammalian species are selected from the group consisting of: ovine, bovine, equine or rodents.

16. The process according to claim 13, wherein the non-human mammalian specie is bovine.

17. Non human high-genetic value mammalian embryos of predetermined sex obtainable according to the process as in claims 1-16.

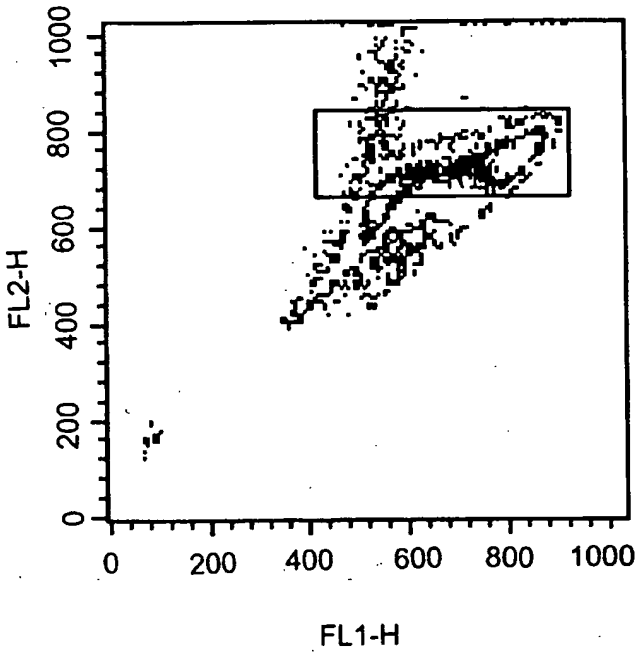

Figure 1

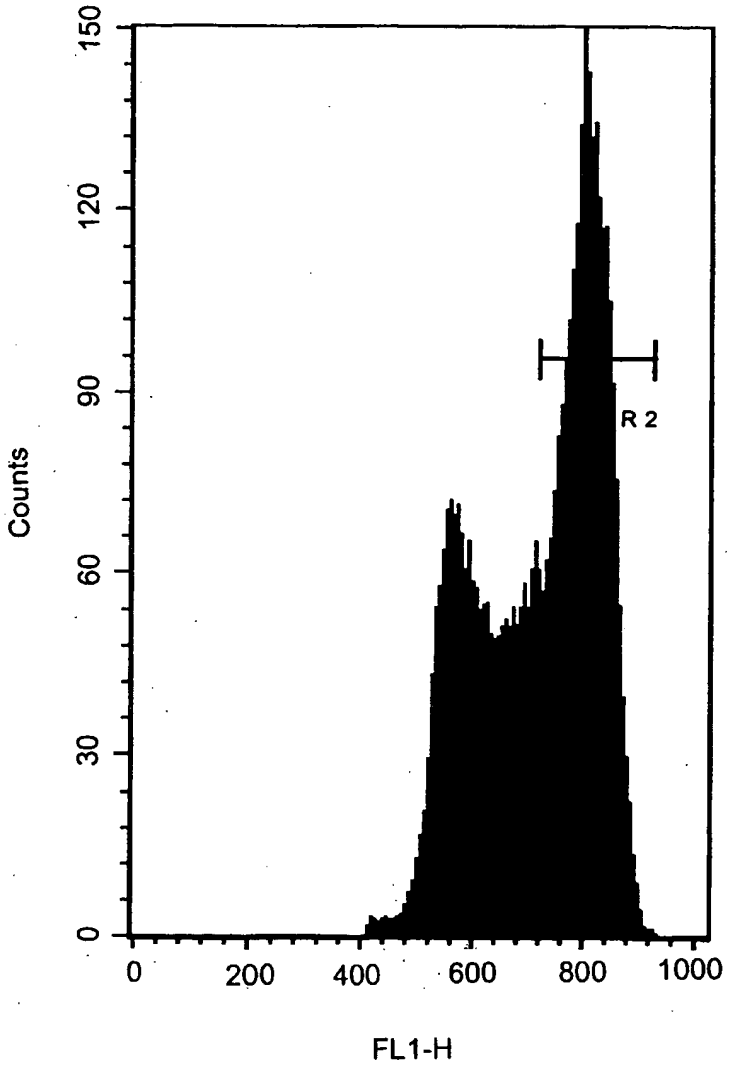

Figure 2

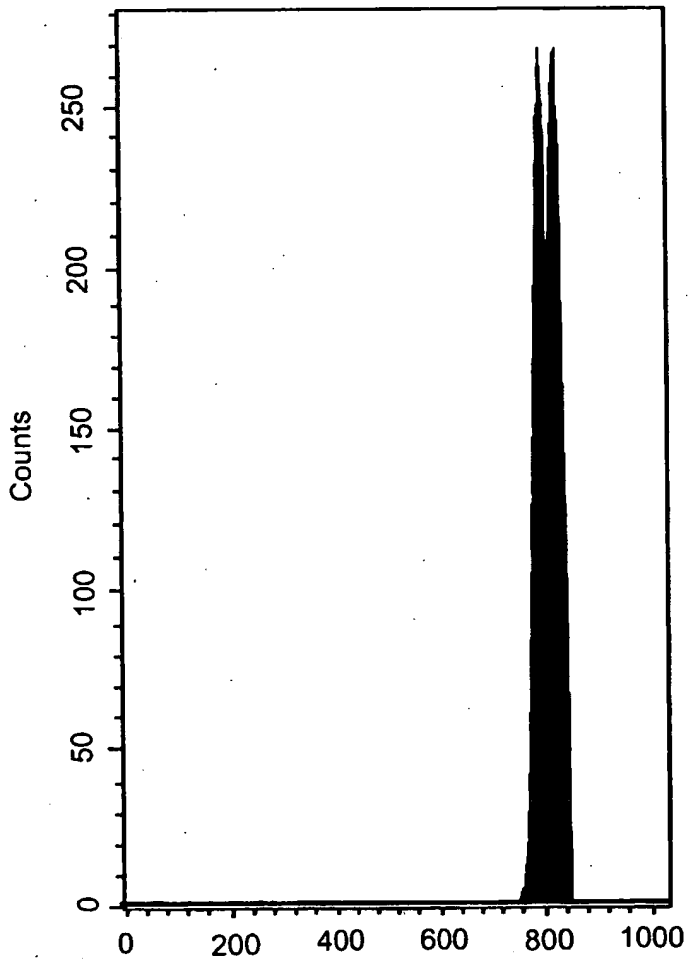

Figure 3

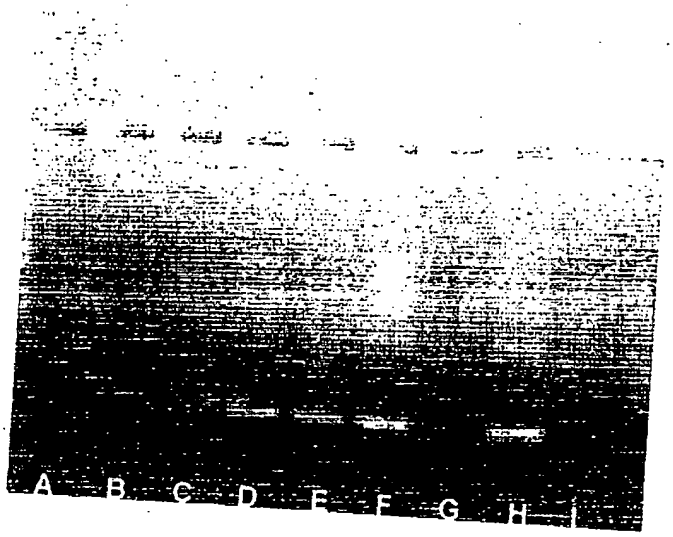

Figure 4

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 01/00281

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 A61D19/00 A01K67/02 G01N33/483 A61K35/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61D A01K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STAP JAN ET AL: "Improving the resolution of cryopreserved X- and Y-sperm during DNA flow cytometric analysis with the addition of Percoll to quench the fluorescence of dead sperm." JOURNAL OF ANIMAL SCIENCE, vol. 76, no. 7, July 1998 (1998-07), pages 1896-1902, XP000985715 ISSN: 0021-8812 the whole document ---	1-17
X	SEIDEL G E JR ET AL: "Insemination of heifers with sexed sperm." THERIOGENOLOGY, vol. 52, no. 8, December 1999 (1999-12), pages 1407-1420, XP000995617 ISSN: 0093-691X the whole document ---	1-17

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 2001	03/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 01/00281

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with Indication,where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO 99 33956 A (XY INC (US)) 8 July 1999 (1999-07-08) page 13, line 19 -page 14, line 14	1-17
Y	RENS WIM ET AL: "Improved flow cytometric sorting of X- and Y-chromosome bearing sperm: Substantial increase in yield of sexed semen." MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 52, no. 1, January 1999 (1999-01), pages 50-56, XP000996007 ISSN: 1040-452X page 51, paragraph 4	1-17
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Y	LU K H ET AL: "In vitro fertilization with flow-cytometrically-sorted bovine sperm." THERIOGENOLOGY, vol. 52, no. 8, December 1999 (1999-12), pages 1393-1405, XP000995618 ISSN: 0093-691X the whole document	1-17

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Information on patent family members

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